

FORM PTO-1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1721-27
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/831019 Unknown
INTERNATIONAL APPLICATION NO. PCT/FR00/02443	INTERNATIONAL FILING DATE 5 September 2000	PRIORITY DATE CLAIMED 6 September 1999
TITLE OF INVENTION METHOD FOR DETECTING AND PURIFYING TCD8+ LYMPHOCYTE POPULATIONS, SPECIFIC OF PEPTIDES PRESENT IN THE CONTEXT OF HLA		
APPLICANT(S) FOR DO/EO/US LANG et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The U.S. has been elected by the expiration of 19 months from the priority date (Article 31). 5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). <ol style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 		
Items 11 To 20 below concern document(s) or information included:		
<ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information. PTO-1449/ International Search Report/ PCT Request/ a front page of the published application 		

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.51) 09/831019		INTERNATIONAL APPLICATION NO. PCT/FR00/02443		ATTORNEY'S DOCKET NUMBER 1721-27	
21. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5): -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO\$1000.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO.....\$710.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div> Surcharge of \$130.00 for furnishing the oath or declaration later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).				\$	860.00
				\$	130.00
CLAIMS		NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims		13	-20 = 0	X	\$18.00
Independent Claims		1	-3 = 0	X	\$80.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					\$270.00
TOTAL OF ABOVE CALCULATIONS =				\$	990.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	0.00
SUBTOTAL =				\$	990.00
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).				\$	0.00
TOTAL NATIONAL FEE =				\$	990.00
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property				\$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application (\$1240.00 - Small Entity = \$620.00)				\$	0.00
TOTAL FEES ENCLOSED =				\$	990.00
				Amount to be:	
				refunded	\$
				Charged	\$
<p>a. <input checked="" type="checkbox"/> A check in the amount of \$990.00 to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.</p> <p>d. <input checked="" type="checkbox"/> The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.</p> <p>NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p> <p>SEND ALL CORRESPONDENCE TO:</p> <p>NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8th Floor Arlington, Virginia 22201-4714 Telephone: (703) 816-4000</p>					
				SIGNATURE	
				B. J. Sadoff	
				NAME	
				36,663	
				May 4, 2001	
				REGISTRATION NUMBER Date	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

LANG et al.

Atty. Ref.: 1721-27

U.S. National Phase of PCT/FR00/02443
Serial No. Unknown

Group:

Filed: May 4, 2001

Examiner:

For: METHOD FOR DETECTING AND PURIFYING TCD8+ LYMPHOCYTE
POPULATIONS, SPECIFIC OF PEPTIDES PRESENT IN THE CONTEXT OF HLA

* * * * *

May 4, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Preliminarily amend the above-identified application as follows:

IN THE CLAIMS

Amend the claims as follows:

3. (Amended) Multimers according to claim 1, characterized in that the modification corresponds to a mutation in the $\alpha 3$ domain of at least one amino acid, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

4. (Amended) Multimers according to claim 1, characterized in that the modification corresponds to chemical modification of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

5. (Amended) Multimers according to claim 1, characterized in that the modification

corresponds to the deletion of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

6. (Amended) Multimers according to claim 1, characterized in that they are in the form of complexes with antigenic peptides.

8. (Amended) Use of multimers according to claim 6 for the purpose of detection and/or isolation of peptide-specific CD8+ T lymphocyte populations.

10. (Amended) Method for the detection of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with multimers complexed with antigenic peptides according to claim 6 under conditions which allow interaction between the modified class I MHC/peptide complexes and T lymphocyte receptors which have an affinity for the said complexes,

- visualization of the lymphocyte populations which are bound to the said complexes.

11. (Amended) Method for isolation of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with magnetic beads on which are bound the peptide/class I CMH analogue complexes according to claim 6 under conditions which allow interaction between the said complexes and T lymphocyte receptors which have an affinity for the said complexes,

- recovery of the bound populations, the screening operation being repeated, if desired, and/or followed, where appropriate, by a stage

- of *in vitro* amplification of the populations selected.

LANG et al.
U.S. National Phase of PCT/FR00/02443
U.S. Serial No. Unknown

12. (Amended) Lymphocyte populations which have been selected and, where appropriate, amplified, characterized in that they are made up exclusively of T lymphocytes which are reactive towards the peptide of a complex with multimers according to claim 6.

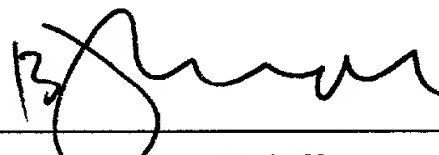
REMARKS

Claims 1-13 are pending. The claims have been amended to delete multiple dependencies, without prejudice. An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



B. J. Sadoff
Reg. No. 36,663

BJS:ms
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

3[]. (Amended) Multimers according to claim 1 [or 2], characterized in that the modification corresponds to a mutation in the $\alpha 3$ domain of at least one amino acid, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

4[]. (Amended) Multimers according to claim 1 [or 2], characterized in that the modification corresponds to chemical modification of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

5[]. (Amended) Multimers according to claim 1 [or 2], characterized in that the modification corresponds to the deletion of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

6[]. (Amended) Multimers according to [any one of claims 1 to 5] claim 1, characterized in that they are in the form of complexes with antigenic peptides.

8[]. (Amended) Use of multimers according to claim 6 [or 7] for the purpose of detection and/or isolation of peptide-specific CD8+ T lymphocyte populations.

10[]. (Amended) Method for the detection of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with multimers complexed with antigenic peptides according to claim 6 [or 7] under conditions which allow interaction between the modified class I MHC/peptide complexes and T lymphocyte receptors which have an affinity for the said complexes,

- visualization of the lymphocyte populations which are bound to the said complexes.

11[7]. (Amended) Method for isolation of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with magnetic beads on which are bound the peptide/class I CMH analogue complexes according to claim 6 [or 7] under conditions which allow interaction between the said complexes and T lymphocyte receptors which have an affinity for the said complexes,

- recovery of the bound populations, the screening operation being repeated, if desired, and/or followed, where appropriate, by a stage

- of *in vitro* amplification of the populations selected.

12[7]. (Amended) Lymphocyte populations which have been selected and, where appropriate, amplified, characterized in that they are made up exclusively of T lymphocytes which are reactive towards the peptide of a complex with multimers according to claim 6 [or 7].

Means for detection and purification of CD8+ T lymphocyte populations specific to peptides presented in the context of HLA

5 The invention relates to means for detection and purification of CD8+ T lymphocyte populations specific to peptides presented in the context of HLA.

10 The T lymphocyte carries a receptor specific to the antigen against which it is directed, called TCR. This TCR is composed of several chains, the α and β chains of which are involved in the specific recognition of a particular antigenic peptide presented in an HLA molecule. This recognition is shown by the ability of the α/β TCR of the T lymphocyte to
15 bind with a certain affinity to HLA-peptide complexes present on the surface of the target cell. Reciprocally, soluble HLA-peptide complexes are capable of binding to the TCR present on the surface of T lymphocytes specific to the HLA-peptide complex in question.

20 At the present time, the system studied most at the molecular level is recognition by CD8+ T lymphocytes of antigenic peptides present in class I major histocompatibility complex (MHC) molecules, and in particular in the HLA-A0201
25 allele.

In this system, it has been established that the affinity of the TCR for the HLA-peptide complex is very low compared to the affinity of an antibody for its antigen. For this reason,
30 detection of TCR-carrier lymphocytes which are reactive towards a specific peptide in this HLA context with the aid of soluble HLA-A0201 molecules which are charged with peptides and marked is impossible. To overcome this low affinity,

Altman et al (1) prepared a multivalent reagent composed of HLA-A0201-peptide complexes where the heavy chain of the HLA is biotinylated, which allows combination as a tetramer with streptavidine. This HLA-peptide tetramer has an increased avidity for the appropriate TCR-carrier T lymphocytes and can therefore be used to visualize reactive populations by immunofluorescence.

However, the TCR is not the only molecule of the T lymphocyte which can interact with the HLA-peptide complex. In fact, during physiological recognition the binding of the TCR to the MHC-peptide complex is intensified by binding of the co-receptor CD8 to a constant portion of class I MHC molecules. The participation of CD8 in the interaction varies from one lymphocyte clone to the other and in some cases can lead to a very significant increase in the capacity for binding to a given HLA-peptide complex. This ability of CD8 to bind to class I HLA consequently leads to a background noise of binding of class I HLA tetramers on the CD8+ T lymphocytes which carry TCR which are non-specific to the HLA peptide complex. This background noise increases with the concentration of tetramer used and can lead to falsely positive immunofluorescence results. To attempt to reduce this non-specific marking, the majority of teams carry out their marking with class I HLA tetramers in the presence of anti-CD8 antibodies. However, only some anti-CD8 antibodies are effective and the optimum concentration ratios between the antibody and the tetramer must be readjusted for each test. As a result of these disadvantages, detection of specific subpopulations with a low representation within a non-specific population (for example of the order of 0.1 to 1%) becomes difficult.

Another potential application of HLA tetramers has moreover been proposed. This comprises isolation by screening (in flow cytometry or by immunomagnetic screening) of lymphocyte populations which are reactive towards a given HLA-peptide complex for the purpose of in vitro expansion and then therapeutic use within passive anti-viral or anti-tumoral immunization protocols. However, the background noise of binding of the tetramer due to the participation of CD8 may constitute a serious obstacle in this application, since it leads to isolation of an often significant fraction of T lymphocytes which are not reactive with respect to the selecting HLA-peptide complex.

Salter et al (2) have shown that binding of a membrane HLA expressed by cells transfected with a CD8 $\alpha\alpha$ co-receptor was modified when the HLA carried a mutation in the $\alpha\beta$ domain.

Study of such mutations by the inventors has led them to verify that soluble mutated tetramers effectively bind less CD8, regardless of whether $\alpha\alpha$ or $\alpha\beta$, combined or not combined with a TCR on the surface of the T lymphocyte, which manifests itself in a reduction in the background noise.

It is therefore to be expected that the loss in affinity resulting from the mutation leads to a loss in the specific signal which is total or restricted to certain CD8-dependent T lymphocyte clones. In this respect, the article by Salter et al shows that certain CD8-dependent alloreactive clones lose their cytotoxicity with respect to cells carrying mutated HLA-A2, whereas others are less affected.

It would thus be possible that the mutated tetramers detect only a fraction of reactive cells (the less CD8-

dependent) within a polyclonal population.

The numerous comparative markings of polyclonal populations with mutated and native tetramers carried out by the inventors by double-marking with an anti-CD8 antibody demonstrate that, on the contrary, the mutated tetramer unexpectedly recognizes the same percentage of specific cells as the native tetramer.

Furthermore, comparison of the marking with the mutated tetramer on a highly CD8-dependent clone and a clone of low CD8 dependency shows a comparable effectiveness of the binding of the tetramer with respect to the intensity of the expression of the TCR.

It thus seems that the mutation very significantly reduces binding of the tetramer to CD8 alone, but affects its binding to the TCR-CD8 complex much less.

The invention therefore lies in the utilization of the properties demonstrated in mutated or, more generally, modified HLA multimers and provides such multimers and their complexes with antigenic peptides, as new products.

It also provides the use of these molecules for the detection and/or isolation of peptide-specific CD8+ T lymphocyte populations.

It additionally provides a method for detection and/or isolation of such populations with the aid of such molecules which are charged with peptide, in particular for applications in diagnostics and therapeutics.

The multimers according to the invention are built up from recombinant protein analogues of class I MHC and are characterized in that the proteins comprise at least one modification in the zone of interaction of a heavy chain of the class I MHC with the CD8 co-receptor of T lymphocytes, leading to a reduction, or even suppression of the affinity of the interaction between the heavy chain and the CD8.

The modification of the zone of interaction more specifically relates to the $\alpha 3$ domain of the heavy chain.

More particularly, it is a mutation in the $\alpha 3$ domain of at least one amino acid with respect to the corresponding domain of a native heavy chain which is capable of binding to the said CD8 co-receptor.

There may be mentioned by way of example the mutation of an alanine residue into a valine residue in position 245 of the $\alpha 3$ domain of the HLA-A2 molecule.

The modification may also consist of a chemical modification of at least one amino acid and/or a deletion of at least one amino acid, this or these types of modification being in addition to one or more mutations, where appropriate.

The invention also provides, as new products, complexes built up from the multimers defined above and antigenic peptides.

In these complexes the multimers are present in particular in the form of tetramers.

According to the invention, these complexes are used for the detection and/or isolation of CD8⁺ T lymphocyte

populations which recognize the antigenic peptide of the complexes in a specific manner.

5 The use of the complexes defined above enables the background noise of non-specific binding to be reduced very significantly without modifying the specific marking and, in addition, removes the need for conjoint use of an anti-CD8 antibody during immunofluorescence analyses.

10 In a preferred use, the said complexes are used in a cell screening process, such as immunomagnetic screening.

15 An immunomagnetic screening technique for isolating specific T lymphocytes in mice is described by Luxembourg et al. (5).

20 This technique is based on the use of a system of beads coated with MHC-peptide complexes (produced in a *Drosophila* system; charged with peptides and chemically biotinylated).

25 The invention also provides a method for detection and/or isolation of peptide-specific CD8+ T lymphocyte populations from a polyclonal population. The detection method is characterized in that it comprises:

- 30 - bringing the polyclonal population into contact with multimers complexed with antigenic peptides as defined above under conditions which allow interaction between the modified class I MHC complexes/peptides and T lymphocyte receptors which have an affinity for the said complexes,
- visualization of lymphocyte populations which are bound to the said complexes.

The visualization is carried out, for example, by

fluorescence using multimers comprising fluorescent compounds.

The method for isolation of peptide-specific lymphocyte populations from polyclonal populations also falls within the context of the invention and can be used, where appropriate, after the above detection stage.

This method utilizes the immunomagnetic screening technique and is characterized in that it comprises:

- bringing the polyclonal population into contact with magnetic beads on which are bound peptide/class I MHC analogue complexes as defined above under conditions which allow interaction between the said complexes and T lymphocyte receptors which have an affinity for these complexes,

- recovery of the bound populations, the screening operation being repeated, if desired, and/or followed, where appropriate, by a stage

- of *in vitro* amplification of the selected populations.

The increase in the differential between the background noise and specific marking with the modified multimers as defined above allows better discrimination of specific lymphocyte populations within a polyclonal population, and therefore makes immunomagnetic screening with these multimers very effective.

The MHC molecules comprise, for example, an enzymatic biotinylation moiety on the heavy chain. The beads on which the complexes are bound are coupled to streptavidine.

The polyclonal populations originate from samples taken from patients, such as synovial fluids or mononucleated cells of peripheral blood.

The amplification stage of selected populations is advantageously carried out by polyclonal stimulation under conditions which do not affect the representativeness of the amplified populations. PHA, IL2 or irradiated PBL, for example, are used.

The invention also provides T lymphocyte populations which have been selected and, where appropriate, amplified, characterized in that they are made up exclusively of T lymphocytes which are reactive towards the peptide of a given complex.

Such peptide-specific populations are of great interest in therapeutics, and more specifically for applications which rely on their re-administration in accordance with adoptive immunotherapy.

The invention thus provides pharmaceutical compositions, characterized in that they are developed from a peptide-specific T lymphocyte population as defined above in combination with a pharmaceutical vehicle.

Such compositions can advantageously be administered by injection.

It is thus possible to restore an antiviral or antitumoral immunity after injection of T lymphocytes which recognize, respectively, a defined class I MHC/viral or tumoral peptide complex, or to correct an immunity disequilibrium (for example the case of autoimmunity) by the administration of T cells directed against a given antigen and having activatory or inhibitory properties on the immune

response.

Other characteristics and advantages of the invention are given in the examples which follow, which are given purely by way of illustration and in which reference is made to figures 1 to 7, which show, respectively

- figures 1 and 2 show the fluorescence intensity (averages) as a function of the concentrations of native tetramers (1A, 2A) and of corresponding mutated tetramers (1B, 2B) with tetramers charged with peptide originating from BMLF1 (fig. 1) or pp65 (fig. 2),

- figure 3 shows the average fluorescence intensities as a function of the concentrations of native and mutated tetramers with specific clones,

- figure 4A shows the percentage of lysis, with or without anti-CD8 antibodies, of 2 specific clones of a B HLA-A0201 line charged with a BMLF1 peptide, and figures 4A and 4B show the results of the marking of these clones,

- figure 5 shows the results of single (with phycoerythrin, PE) and double (PE and marked anti-CD8 antibodies) marking of native and mutated tetramers with the lymphocytes of 2 patients and

- figures 6 and 7 show the results of immunomagnetic screening carried out with native tetramers and mutated tetramers.

Example 1 : Preparation of an HLA-A0201 tetramer mutated in the zone of interaction with the CD8 co-receptor.

A bacterial expression plasmid containing the cDNA which codes for the heavy chain of HLA-A0201, lengthened by a sequence which codes for an enzymatic biotinylation motive was used (construction according to Altman et al (1)). The

zone which codes for the $\alpha 3$ domain had been amplified with the specific primers SEQ ID no. 1

5' CCTTCCAGAAGTGGGTGGCTGTGGTGGTGCC 3'

5 and SEQ ID no. 2

5' GGCACCACCACAGCCACCCACTTCTGGAAGG 3'

10 A mutation of a base to transform the alanine codon into a valine codon was introduced into the amplification fragments using the Stratagene QuickChange Site-directed Mutagenesis R kit.

15 The mutated fragment was reintroduced into the expression plasmid and the presence of the mutation was checked by sequencing.

20 The mutated HLA-A0201 heavy chain had been produced in the bacterial inclusion body and the HLA monomer charged with peptide, and then the corresponding mutated tetramer, could be obtained in accordance with a renaturation protocol previously described by Garboczi et al (3).

25 Example 2 : Comparative study of the effectiveness and specificity of immunofluorescent marking between a native HLA-A0201 tetramer and the mutated HLA-A0201 tetramer charged with different peptides

1) Marking of lymphocyte clones

30 Native HLA-A0201 tetramers and corresponding mutated tetramers were charged either with a peptide originating from the protein BMLF1 of the EBV virus or with a peptide originating from the protein pp65 of CMV.

The native tetramers are obtained in accordance with the technique of example 1, but without the mutation.

Specific and non-specific clones were marked and used at increasing concentrations.

Figure 1 gives the average fluorescence intensity (AFI) obtained with the native HLA-A2/BMLF1 tetramers (figure 1A) and with the corresponding mutated tetramers (figure 1B).

It is found that the native HLA-A2/BM tetramer shows a background noise of binding on some non-specific clones which increases with the dose of tetramer used.

This background noise does not seem associated exclusively with the HLA restriction of the clone in question, but also depends on the peptide charged, since two HLA-A0201-restricted anti-IE1 clones give a high background noise, whereas the similarly HLA-A0201-restricted anti-melan-A clone gives a moderated background noise.

With the mutated tetramer, the averages of the fluorescence (AFI) obtained on the specific clones (BM/A2) are lower than those obtained with the native tetramer, but the background noise on the non-specific clones is virtually zero, regardless of the concentration of tetramer used.

It can be seen that this difference in background noise between the native tetramer and the mutated tetramer is not peculiar to the A2/BM tetramer, since it is also observed with the HLA-A2/pp65 tetramer (see figure 2).

In this figure 2, the average fluorescence is indicated

- 12 -

on a log scale in order to visualize the low background noise of marking obtained with the mutated tetramer. It is found that the differential between the specific marking and the non-specific marking is of the order of $2 \log (10^2)$ with the mutated A2/pp65 tetramer, whereas it is only 1 log with the native tetramer. The two notable exceptions are the two anti-EBV CD4 clones, for which the background noise is very low with the native tetramer. This observation supports the hypothesis according to which the background noise of marking obtained with the native tetramer is due to a certain percentage of non-specific binding of the tetramer to CD8.

Figure 3 shows the marking differences obtained with the mutated and native tetramers charged with pp65 on specific clones. The difference between the saturation curves obtained with the mutated tetramer and the native tetramer show that there is a greater number of binding sites for the native tetramer than for the mutated tetramer on these clones: it is therefore highly probable that in the case of the native tetramer some valencies interact with the CD8 alone, especially since this molecule is expressed at a greatly higher density than that of TCR on the surface of the T lymphocyte.

Other experiments have been carried out to test whether the specific marking with the mutated tetramer was affected significantly by the degree of CD8-dependence of specific clones. In fact, it is well-known that among the T CD8+ clones, some clones have a high need of CD8 to intensify the specific interaction between their TCR and the HLA-peptide complex, while other clones dispense with it.

The degree of CD8 dependency was estimated by the

WO 01/18053

PCT/FR00/02443

- 13 -

cytotoxicity test of Couedel et al (4) with or without anti-CD8 antibodies and is regarded as inversely proportional to the affinity of the TCR for the HLA-peptide complex.

Figure 4A shows a test of cytotoxicity against an HLA-A0201 line charged with BMLF1 peptide (10 μ M) with two specific clones. The clone A2.10 is highly CD8-dependent since its cytotoxicity is annulled by the anti-CD8 antibody, whereas the A4.5 clone has a relatively low CD8 dependency.

Marking with the mutated tetramer and marking with an anti-CD3 antibody was carried out to estimate the number of TCR expressed on the surface. The results are given in figures 4B (CD3 marking) and 4C (marking with the mutated BMFL1/A2 tetramer).

It is found that the ratio of tetramer marking/CD3 marking is very comparable for the two clones, which indicates that marking with the mutated tetramer is affected little by the degree of CD8 dependency (and thus, by inference, by the TCR affinity).

The reduction in the affinity for CD8 induced by the mutation of the HLA molecule thus does not significantly affect the specific marking of the tetramer.

b) Detection of specific cells within a polyclonal population

To compare the ability of the native and mutated tetramer to detect a small percentage of specific cells within a polyclonal population, peripheral lymphocytes from two HLA-A0201 patients (designated A and B below) were marked. These

are patients in whom an anti-CMV pp65 peptide response was demonstrated beforehand. These lymphocytes were amplified by polyclonal methods in vitro beforehand and frozen.

5 The results obtained for patients A and B are shown in figure 5, figure 5A corresponding to patient A and figure 5B to patient B. They are the results of single marking of HLA-A0201/pp65 tetramers with phycoerythrin in an amount of 20 μ g/ml and corresponding double marking (tetramers marked with
10 PE and anti-CD8 antibodies marked with FITC). This figure also gives the average fluorescence intensity (log).

15 Firstly, it is found that the discrimination ability of the native tetramer with single marking varies according to the percentage of specific cells in the starting population. In fact, for patient A, the peak of positive cells, which represents 5.60%, is easily identifiable, whereas for patient B this peak is contaminated by non-specific marking, which renders it difficult to determine the percentage of positive
20 cells. In agreement with the literature, double marking of native tetramer and anti-CD8 reduces the background noise and therefore allows clear identification of the positive sub-population and precise estimation of its percentage (0.84% in this case). This thus shows that precise estimation of the
25 percentage of positive cells with the native tetramer involves the use of double marking with anti-CD8.

30 On the other hand, single marking with the mutated tetramer allows identification without ambiguity of a peak of positive cells in the two patients. The percentages of positive cells obtained are virtually identical to those obtained with double marking with the native tetramer. This demonstrates that all the specific cells which can be detected

WO 01/18053

PCT/FR00/02443

- 15 -

by the native tetramer are also detectable with the mutated tetramer, and thus corroborates the results obtained with the clones of high CD8 and low CD8 dependency, that is to say the mutation does not significantly affect the specific
5 recognition.

Furthermore, these results show that the double marking with the mutated tetramer does not provide new elements with respect to single marking. Finally, it can be seen that the
10 averages of the fluorescence obtained with the anti-CD8 antibodies with double marking with the native tetramer (216 and 193 for patients A and B) are significantly lower than those obtained with double marking with the mutated tetramer (366 and 370 for patients A and B). The averages of the
15 fluorescence obtained after single marking of the total population with anti-CD8 were, respectively, 340 and 355 for patients A and B. This demonstrates the binding competition between the anti-CD8 antibody and the native tetramer, competition which does not result with the mutated tetramer.

20 The use of the mutated tetramer thus allows double marking with anti-CD8 to estimate the percentage of specific cells within a polyclonal population to be dispensed with.

25 Example 3: Comparative study of the effectiveness and specificity of immunomagnetic screening carried out with the native HLA-A0201 tetramer and the mutated HLA-A0201 tetramer charged with the peptide p65.

30 Biotinylated HLA-A0201 monomers charged with peptide pp65 were bound to magnetic beads coupled to streptavidine (Dynabeads M-280 Streptavidine, DYNAL). The lymphocyte populations used in the study originated from the synovial

fluids or PBL of patients suffering from rheumatoid polyarthrititis or PBL originating from healthy donors seropositive in respect of CMV.

5 The results of single marking with the native tetramer and the mutated tetramer on these polyclonal populations are shown in figure 6. With the mutated tetramer it is possible to demonstrate a low percentage of positive cells in the two samples (0.22% and 0.14%), positive cells which are virtually
10 unidentifiable with the native tetramer.

15 These populations were subjected to screening with beads charged either with the native tetramer or with the mutated tetramer, and the populations selected were then amplified in vitro by polyclonal stimulation (PHA, IL2, irradiated PBL), a stimulation which does not affect the representativeness of the amplified populations.

20 Marking was then carried out on these populations which had been screened and amplified with the two tetramers.

25 As shown in figure 6, the cells originating from screening with the native tetramer are marked positively with this same tetramer, but on the other hand a very low percentage of these cells are positive with the mutated tetramer (0.7% and 2.88%).

30 The results are remarkably different after screening with the mutated tetramer, since the populations which have been screened are highly positive with the two tetramers.

 The concentration factor of positive cells obtained with the mutated tetramer is 421 for sample 1 (92.64% v. 0.22%) and

693 for sample 2 (97.01% v. 0.14%).

These results show that by screening with the mutated tetramer, it is possible to obtain a pure positive population of more than 90% from a sample in which this population represents 0.1 to 0.2%.

The general nature of this observation is illustrated by the table below, which describes the results of the screening carried out with the native tetramer and the mutated tetramer starting from PBL and synovial lymphocytes of patients and healthy donors.

% of positive cells of tetramers (concentration factor)

Samples	Not screened	First screening with		Second screening with	
		A2/pp65 native	A2/pp65 mutated	A2/pp65 native	A2/pp65 mutated
SFL 1	14.0	95.0 (6.8)			
PBL 1	5.6	89.5 (16.0)	97.9 (17.5)		
SFL 2	1.4	25.3 (18.1)		75.9 (3.0)	
PBL 2	0.8	51.1 (63.9)	97.5 (121.9)		
SFL 3	0.6	13.7 (22.8)			
PBL 3	nd	8.5 (nd)		12.0 (1.4)	
SFL 4	0.3	4.1 (13.6)		8.6 (2.1)	
SFL 5	0.2	0.7 (3.5)	92.5 (462.5)		
SFL 6	0.14	2.9 (20.6)	97.1 (693.6)		
PBL 4	0.00		82.9 (921.1)		
PBL 5	0.02	0.04 (2.0)	2.2 (110.0)	98.7	(44.90)

By carrying out repeated screening with the mutated tetramer, it is possible to obtain pure specific populations from sub-populations which are even less frequent (cf. PBL5 in the table). On the other hand, this result is much more difficult to obtain with the native tetramer; it seems in fact that the non-specific cells isolated by a first screening with the native tetramer had an affinity sufficient to be selected again during the subsequent screening. Very mediocre concentration factors are thus arrived at by carrying out a second screening (cf. PBL3 and SFL4).

Another study related to the reactivity of populations which had been subjected to screening to verify that the cells selected for their ability to bind the mutated A2/pp65 tetramer were certainly reactive with respect to this HLA-peptide complex in a physiological context.

To this effect, the activation of screened lymphocyte populations, objectified by induction on the surface of the α chain of the receptor for IL2 (CD25), after placing in the presence of T2 cells (A0201+) charged with peptide pp65, was studied.

As shown in figure 7, the populations which had been screened using the native tetramer do not express CD25 after contact with T2 cells charged with peptides.

On the other hand, the majority of these cells which have been screened with the mutated tetramer become activated in the presence of T2 charged with the peptide pp65 and the percentage of CD25-positive cells corresponds well with the percentage of cells which were marked by the mutated tetramer (90.63% of activated cells v. 92.64% of marked cells and

97.20% v. 97.01% for patients 1 and 2 respectively).

These results demonstrate that the cells marked and screened with the mutated tetramer are exclusively reactive cells, whereas the cells screened with the native tetramer are non-reactive.

The fact that no reactive cell is found after screening with the native tetramer whereas a distinction between some positive cells in this population was achieved is probably due to too low a percentage of these cells for them to be detectable in a functional test.

All of these results demonstrate the manifest superiority of the mutated tetramer with respect to the native tetramer for selection by immunomagnetic screening of populations with a low representation in the starting population.

If the specific populations are more numerous in the starting sample, it thus becomes possible to isolate reactive cells with the native tetramer, but the degrees of purity of the populations obtained are much lower than those obtained in parallel with the mutated tetramer (table).

BIBLIOGRAPHY REFERENCES

- 1) Altman J.D. et al, 1996, Science 274:94-6 and US Patent 5 635 363
- 2) Salter, R.D. et al, 1990, Nature 345:41-46
- 3) Garboczi D.N. et al, 1992, Proc Natl Acad Sci USA 89:3429-33
- 4) Couedel, C., M. et al, 1999, J. Immunol 162 6351 B.
- 5) Luxembourg A.T. et al, Nature Biotechnology, vol. 16, March 1998, 281-285

CLAIMS

1/ Multimers built up from recombinant proteins analogues of class I MHC, characterized in that the proteins comprise at least one modification in the zone of interaction of a heavy chain with the CD8 co-receptor of T lymphocytes leading to a reduction, or even suppression of the affinity of the interaction between the heavy chain and CD8.

2/ Multimers according to claim 1, characterized in that the modification relates to the $\alpha 3$ domain of the heavy chain.

3/ Multimers according to claim 1 or 2, characterized in that the modification corresponds to a mutation in the $\alpha 3$ domain of at least one amino acid, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

4/ Multimers according to claim 1 or 2, characterized in that the modification corresponds to chemical modification of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

5/ Multimers according to claim 1 or 2, characterized in that the modification corresponds to the deletion of at least one amino acid of the $\alpha 3$ domain of a heavy chain, with respect to the corresponding domain of a native heavy chain capable of binding to the said CD8 co-receptor.

6/ Multimers according to any one of claims 1 to 5, characterized in that they are in the form of complexes with antigenic peptides.

7/ Multimers according to claim 6, characterized in that they are in the form of tetramers.

8/ Use of multimers according to claim 6 or 7 for the purpose of detection and/or isolation of peptide-specific CD8+ T lymphocyte populations.

9/ Use according to claim 8 in a process for cell screening, such as immunomagnetic screening.

10/ Method for the detection of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with multimers complexed with antigenic peptides according to claim 6 or 7 under conditions which allow interaction between the modified class I MHC/peptide complexes and T lymphocyte receptors which have an affinity for the said complexes,
- visualization of the lymphocyte populations which are bound to the said complexes.

11/ Method for isolation of peptide-specific CD8+ T lymphocyte populations from a polyclonal population, characterized in that it comprises:

- bringing the polyclonal population into contact with magnetic beads on which are bound the peptide/class I CMH analogue complexes according to claim 6 or 7 under conditions which allow interaction between the said complexes and T lymphocyte receptors which have an affinity for the said complexes,
- recovery of the bound populations, the screening operation being repeated, if desired, and/or followed, where appropriate, by a stage

- of in vitro amplification of the populations selected.

12/ Lymphocyte populations which have been selected and, where appropriate, amplified, characterized in that they are made up exclusively of T lymphocytes which are reactive towards the peptide of a complex with multimers according to claim 6 or 7.

13/ Pharmaceutical compositions which can be used, in particular, in immunotherapy, characterized in that they are built up from a lymphocyte population according to claim 12 in combination with a pharmaceutically inert vehicle.

WO 01/18053

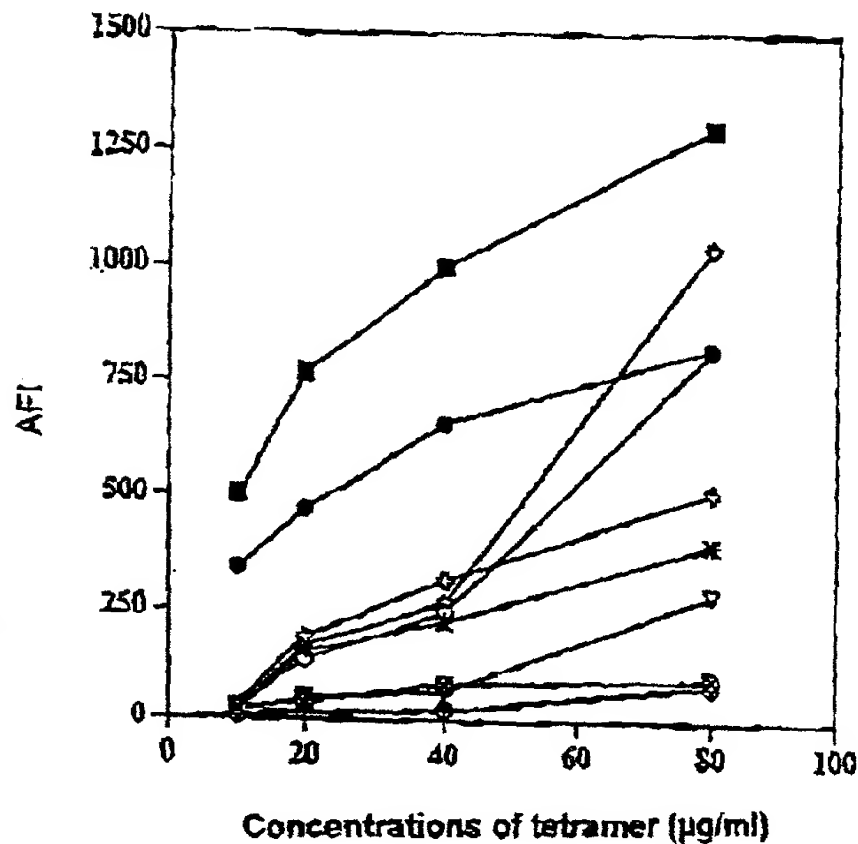
PCT/FR00/02443

1/7

Figure 1A

HLA-A2/BMLF1 tetramer

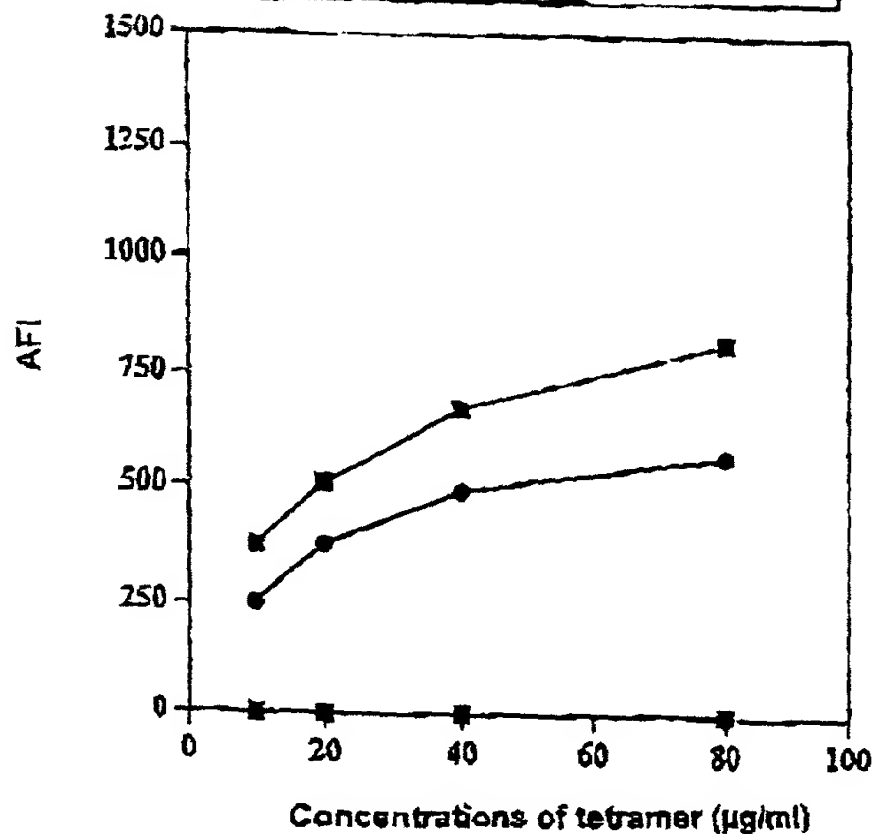
FIGURE 1



- A2.10 BM/A2
- A4.5 BM/A2
- LP16 IE1/A2
- LP23 IE1/A2
- ▽— Melan-A/A2
- ◇— BZ/B18
- *— BZ/B14
- ⊙— Tyrosinase/B35
- 22.19 BZ/B40

Figure 1B

Mutated HLA-A2/BMLF1 tetramer



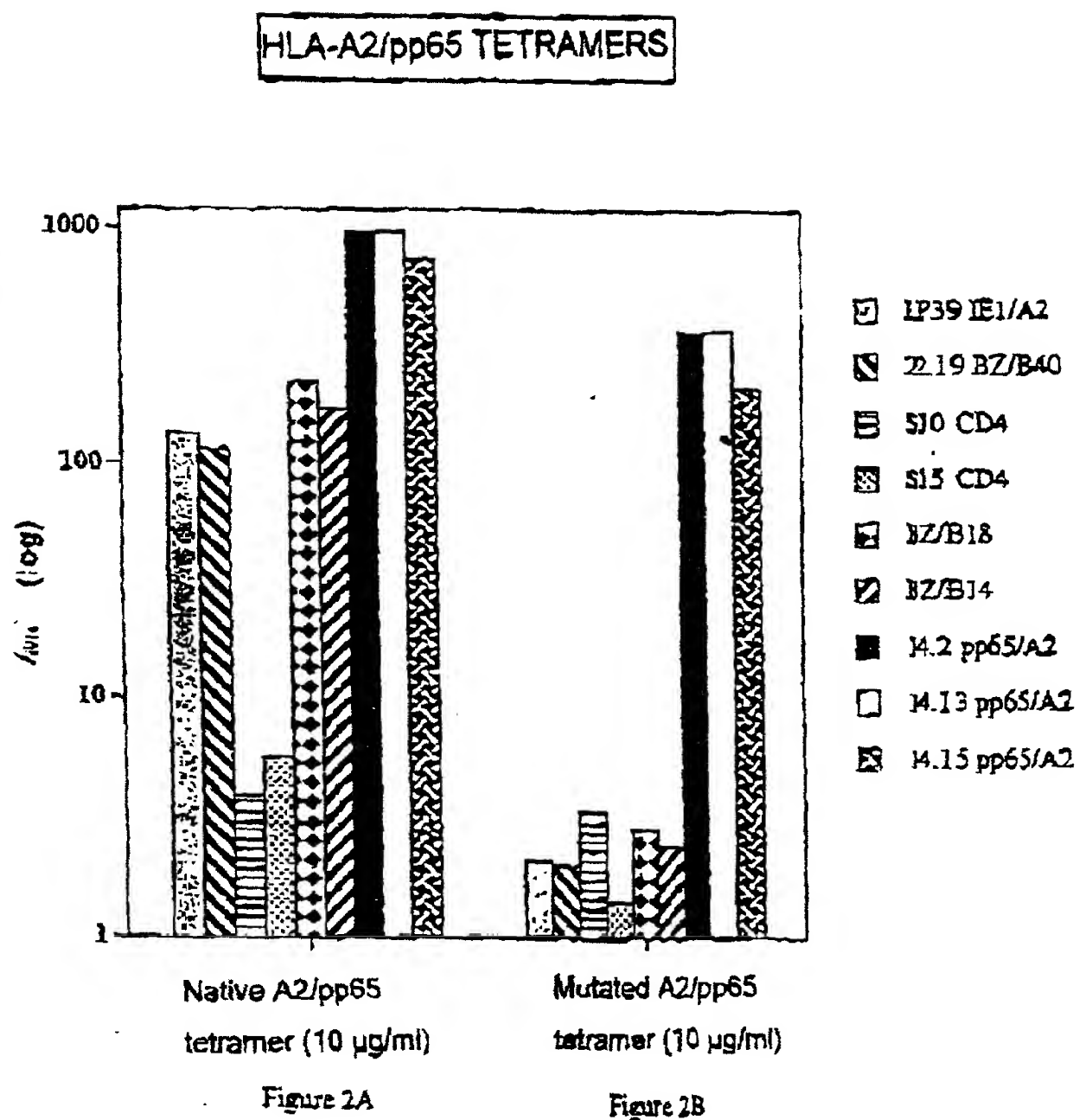
REPLACEMENT PAGE (RULE 26)

WO 01/18053

PCVFR00/02443

2/7

FIGURE 2

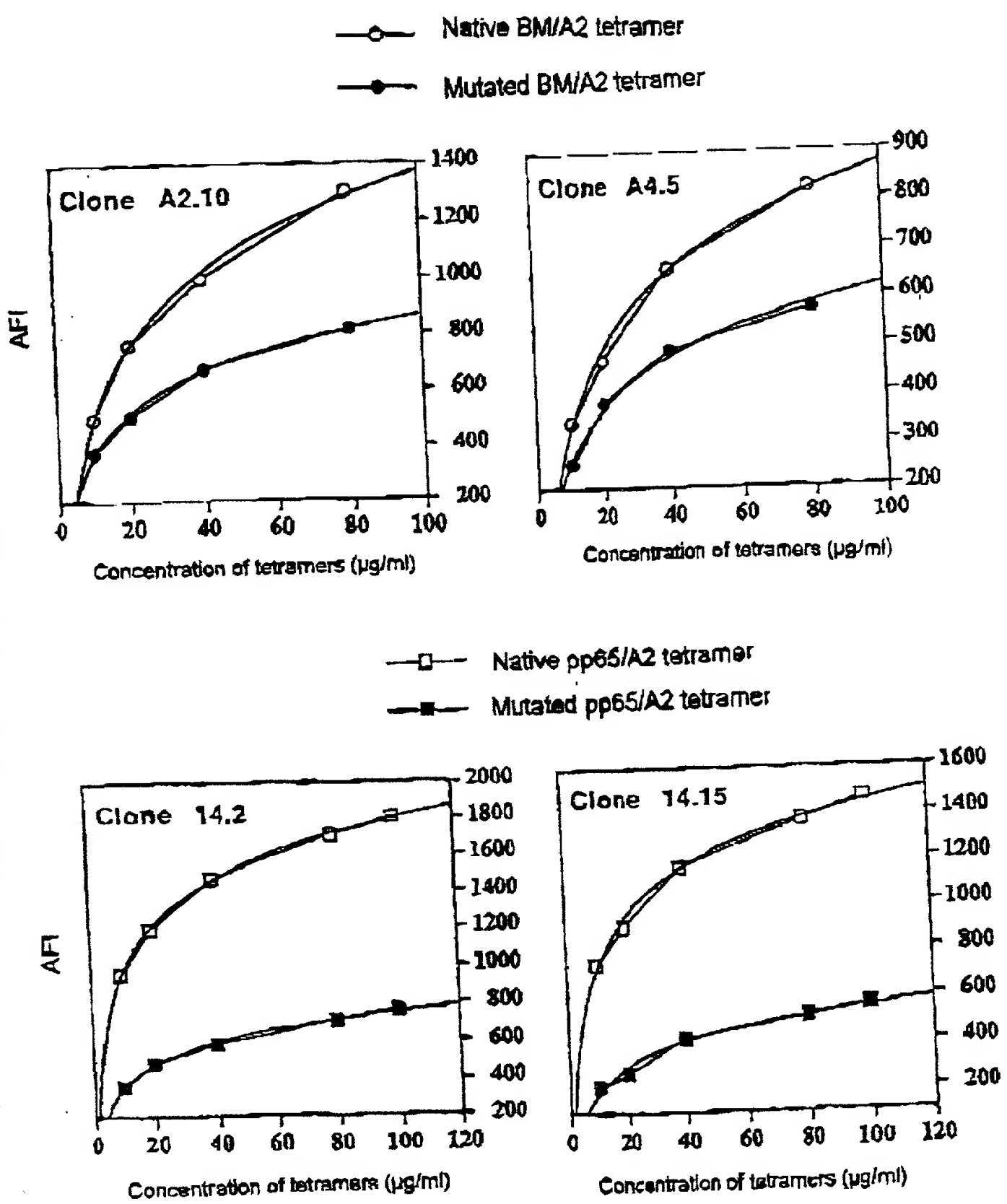


WO 01/18053

PCT/FR00/02443

3/7

FIGURE 3



WO 01/18053

PCT/FR00/02443

4/7

FIGURE 4

FIGURE 4A

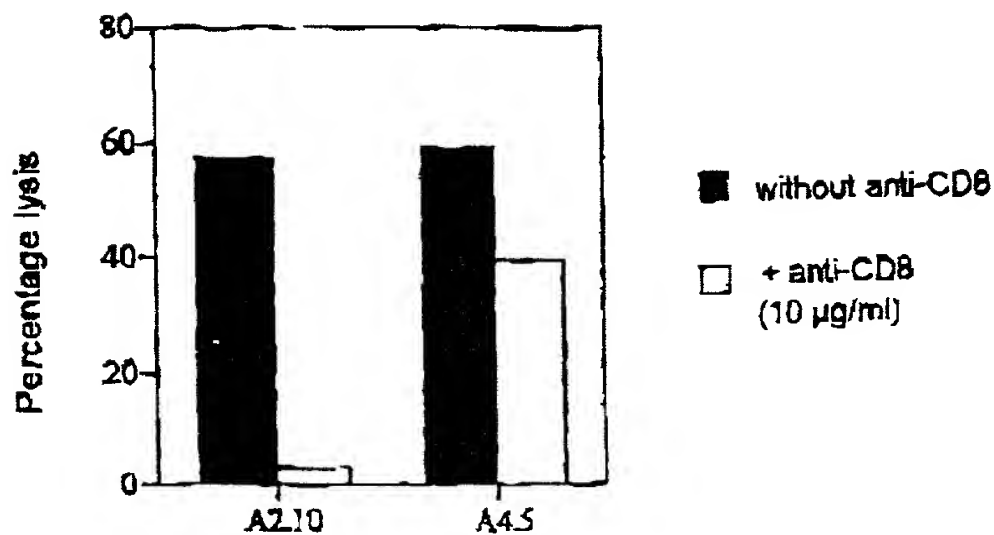


FIGURE 4B

CD3
marking

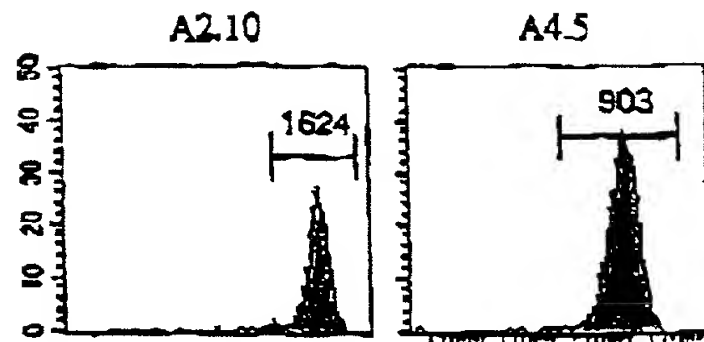
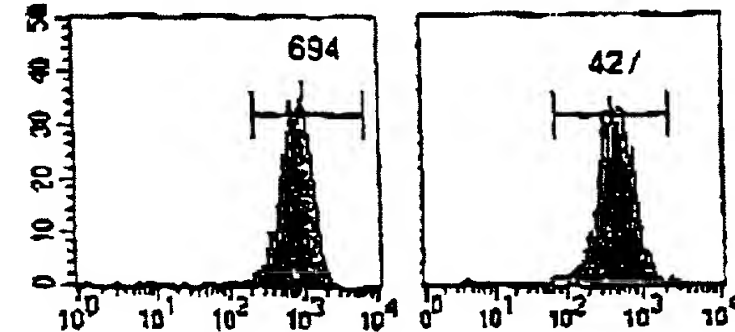


FIGURE 4C

mutated
Bmi1/A2
tetramer
marking



Ratio of mutated tetramer AFI/CD3 AFI

A2.10: 0,43

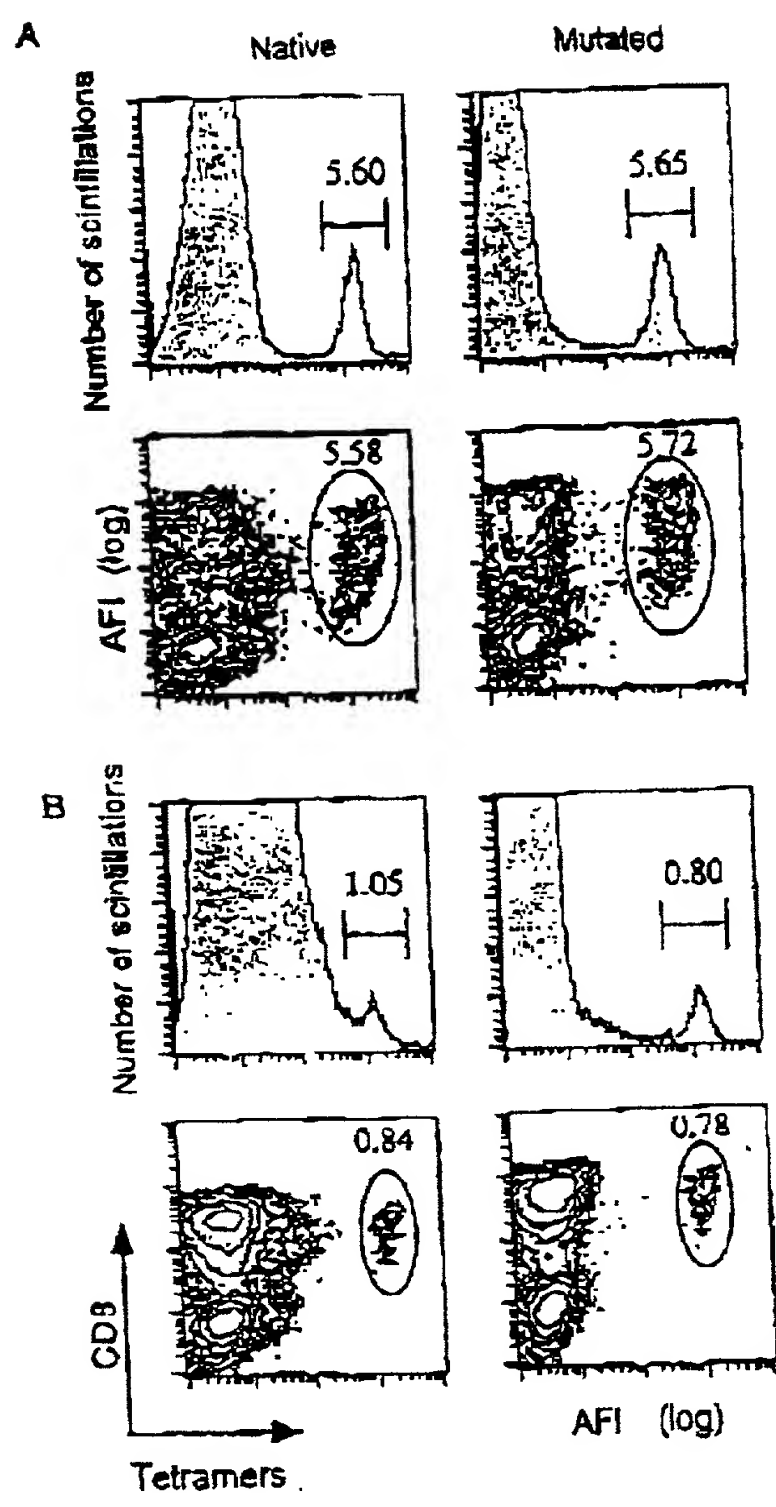
A4.5: 0,47

WO 01/18053

PCT/FR00/02443

FIGURE 5

57



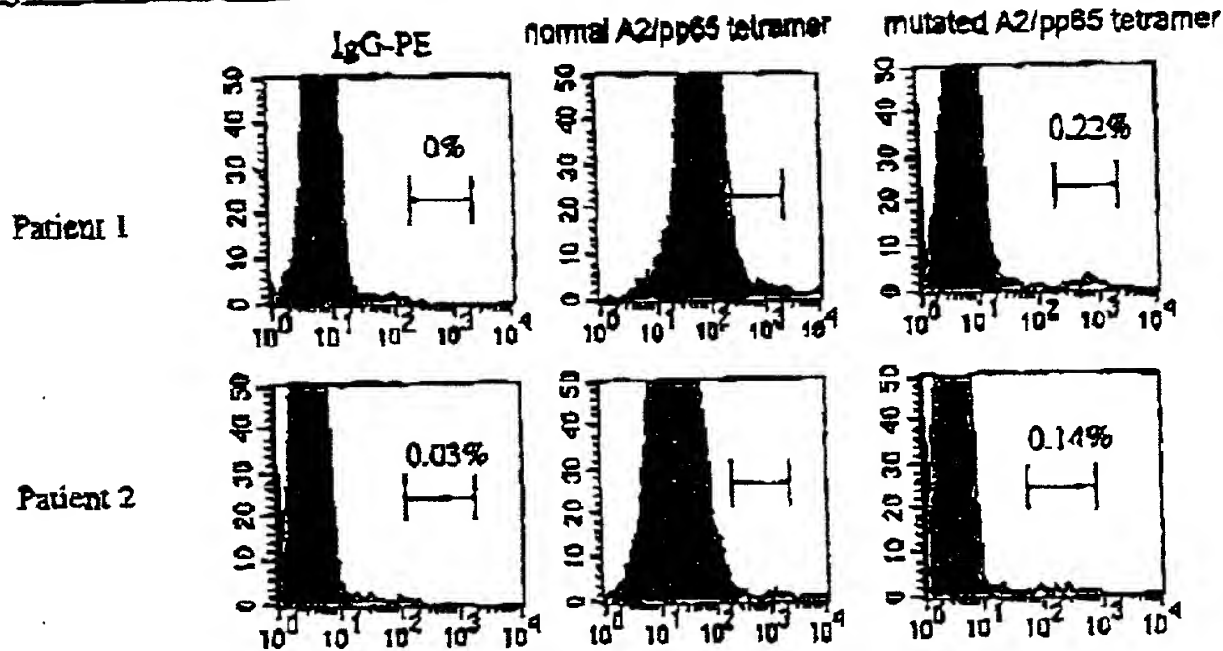
WO 01/18053

PCT/FR00/02443

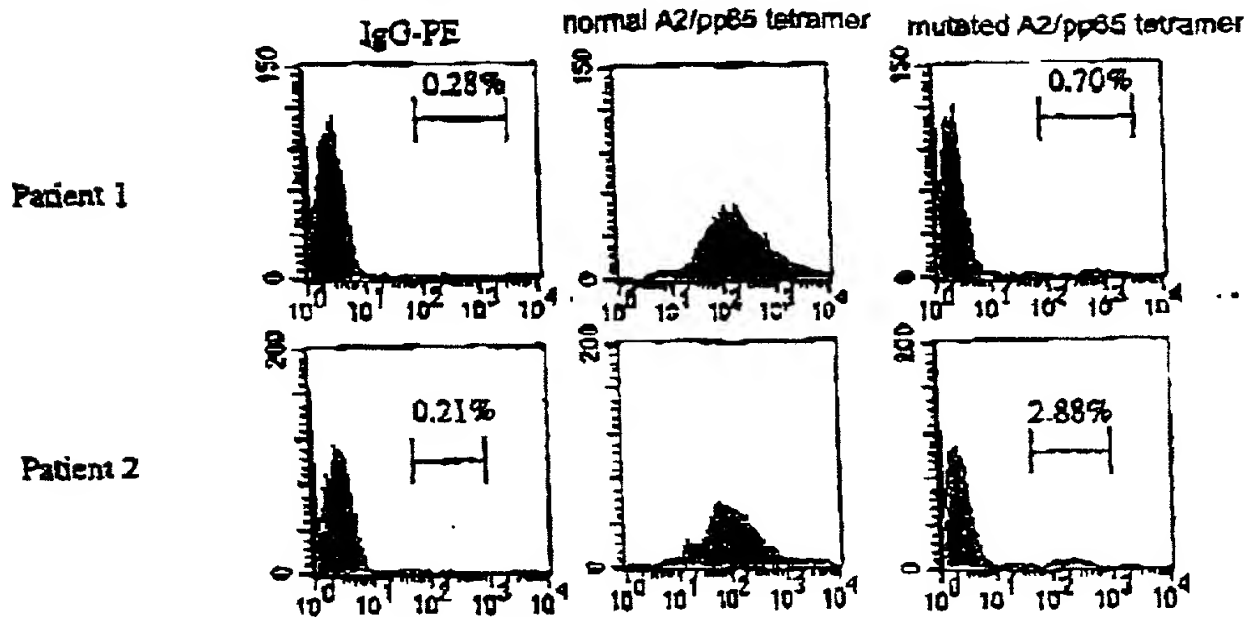
FIGURE 6

Marking before screening

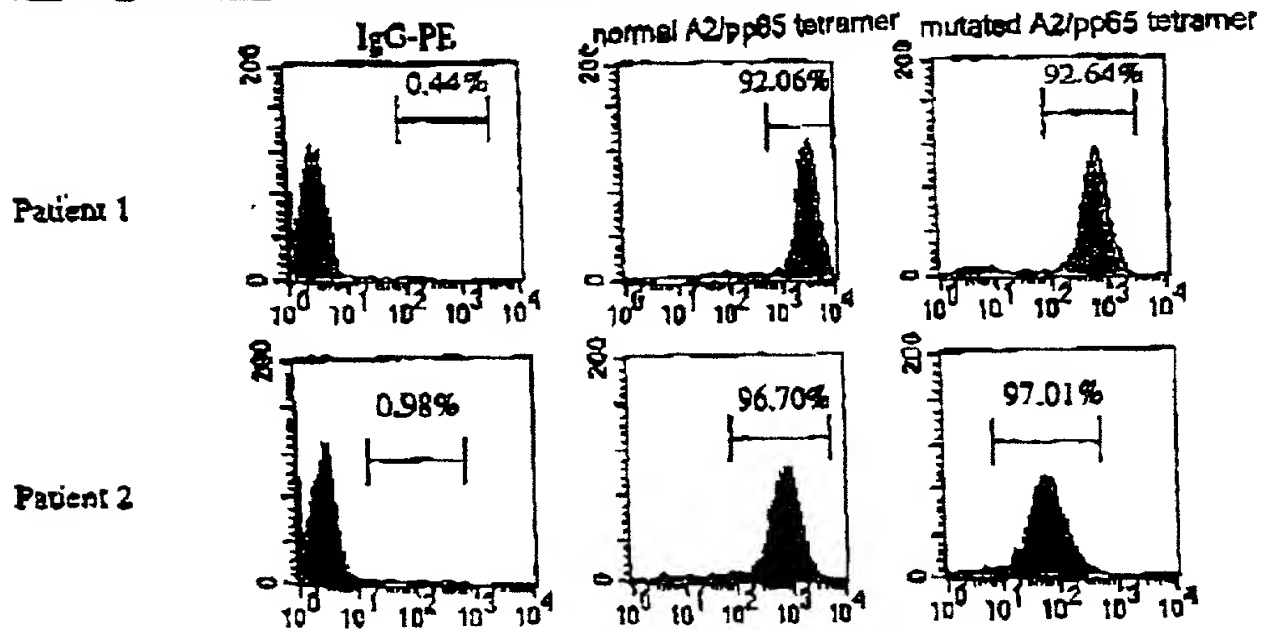
6/7



Marking after screening with normal A2/pp65 tetramer



Marking after screening with mutated A2/pp65 tetramer

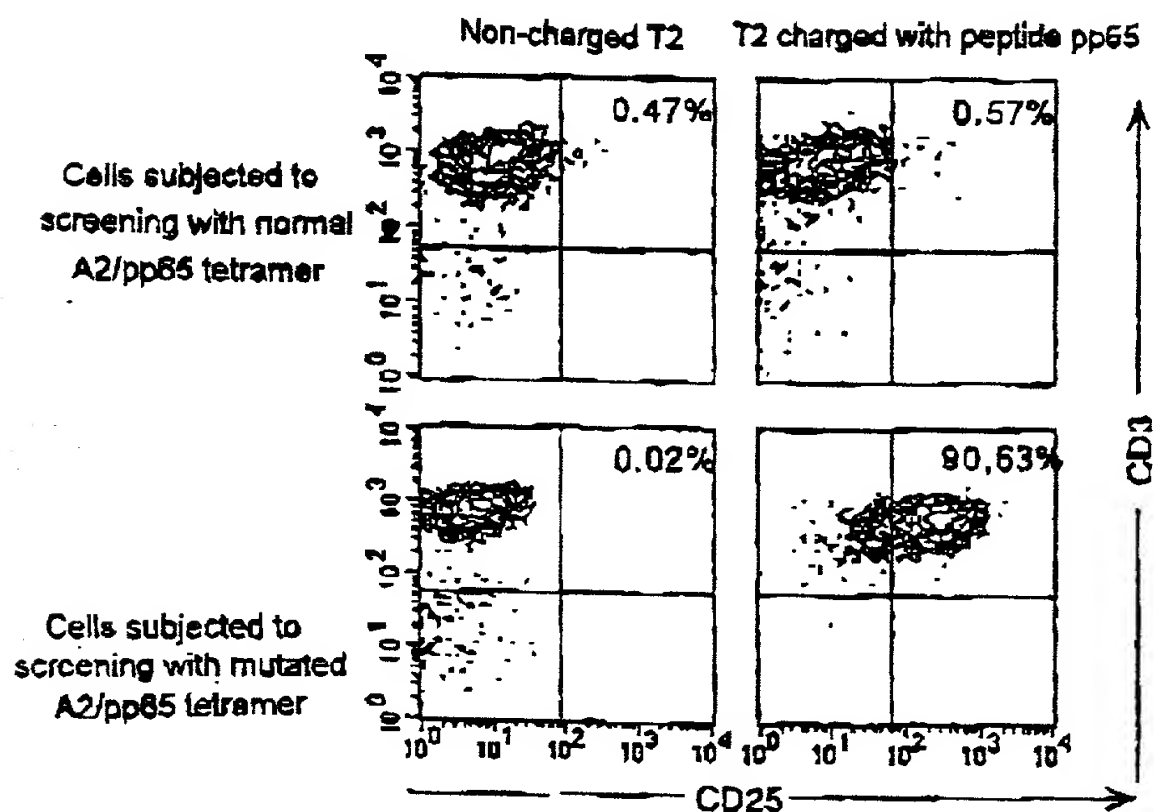
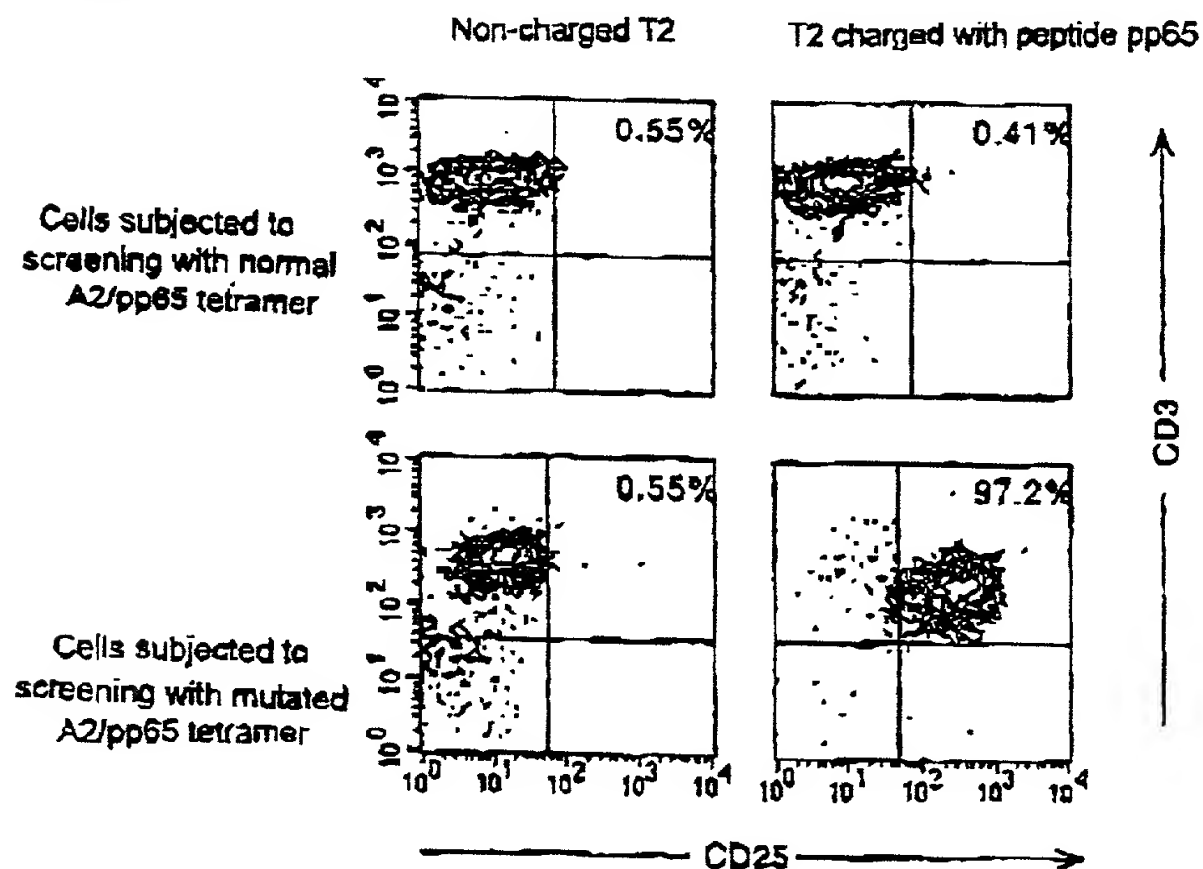


WO 01/18053

PCT/FR00/02443

7/7

FIGURE 7

Patient 1Patient 2

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR DETECTING AND PURIFYING TCD8+ LYMPHOCYTE POPULATIONS, SPECIFIC OF PEPTIDES PRESENT IN THE CONTEXT
OF HLA

the specification of which (check applicable box(es)):

☒ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____ (Atty Dkt. No. 1721-27)
☒ was filed as PCT International application No. PCT/FR00/02443 on 05 09 2000
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
9911133	France	06 09 1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Date/Month/Year Filed
--------------------	-----------------------

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned Pending
PCT/FR00/02443	05 09 2000	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

1. Inventor's Signature: [Signature] Date: 15-06-01
Inventor: François Lang French
(first) (last) (citizenship)
Residence: (city) Nantes (state/country) France
Mailing Address: 9, rue Magnane, Nantes, France
(Zip Code) F-44000

2. Inventor's Signature: [Signature] Date: 15.06.01
Inventor: Marie Bodinier French
(first) (last) (citizenship)
Residence: (city) Nantes (state/country) France
Mailing Address: 53, rue Fauré, Nantes, France
(Zip Code) F-44000

☒ See attached sheet(s) for additional inventor(s) information!!

3. Inventor's Signature: [Signature] Date: 20 fev 2001
Inventor: François Davodeau French
(first) MI (last) (citizenship)
Residence: (city) Nantes (state/country) France
Mailing Address: 9, rue des Capitaines Clerville, Nantes, France
(Zip Code) F-44000

4. Inventor's Signature: [Signature] Date: June 19, 2001
Inventor: Marc Bonneville French
(first) MI (last) (citizenship)
Residence: (city) Vertou (state/country) France
Mailing Address: 60, rue Massonnière, Vertou, France
(Zip Code) F44120

5. Inventor's Signature: _____ Date: _____
Inventor: _____
(first) MI (last) (citizenship)
Residence: (city) _____ (state/country) _____
Mailing Address: _____
(Zip Code) _____

6. Inventor's Signature: _____ Date: _____
Inventor: _____
(first) MI (last) (citizenship)
Residence: (city) _____ (state/country) _____
Mailing Address: _____
(Zip Code) _____

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.